



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/64, 15/86, 15/63	A1	(11) International Publication Number: WO 96/24677 (43) International Publication Date: 15 August 1996 (15.08.96)
(21) International Application Number: PCT/US96/01958 (22) International Filing Date: 8 February 1996 (08.02.96) (30) Priority Data: 08/385,446 8 February 1995 (08.02.95) US (60) Parent Application or Grant (63) Related by Continuation US 08/385,446 (CIP) Filed on 8 February 1995 (08.02.95) (71) Applicant (for all designated States except US): THOMAS JEFFERSON UNIVERSITY [US/US]; 11th & Walnut Streets, Philadelphia, PA 19107 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): ZHANG, Hui [CN/CN]; Apartment 1201, 206 South 13th Street, Philadelphia, PA 19107 (CN). POMERANTZ, Roger, J. [US/US]; 711 Harvest Hill Road, Chalfont, PA 18914 (US). (74) Agents: LICATA, Jane, Massey et al.; Law Offices of Jane Massey Licata, Woodland Falls Corporate Park, Suite 201, 210 Lake Drive East, Cherry Hill, NJ 08002 (US).		(81) Designated States: CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>
(54) Title: A METHOD FOR INCREASING TRANSDUCTION EFFICIENCY OF RECOMBINANT RETROVIRAL VECTORS		
(57) Abstract A method for improving the transduction efficiency of retroviral vectors into a host cell wherein the retroviral vectors are incubated with deoxyribonucleoside triphosphates prior to transduction into the host cell is provided.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic			SE	Sweden
CG	Congo	KR	Republic of Korea	SG	Singapore
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LR	Liberia	SZ	Swaziland
CS	Czechoslovakia	LT	Lithuania	TD	Chad
CZ	Czech Republic	LU	Luxembourg	TG	Togo
DE	Germany	LV	Latvia	TJ	Tajikistan
DK	Denmark	MC	Monaco	TT	Trinidad and Tobago
EE	Estonia	MD	Republic of Moldova	UA	Ukraine
ES	Spain	MG	Madagascar	UG	Uganda
FI	Finland	ML	Mali	US	United States of America
FR	France	MN	Mongolia	UZ	Uzbekistan
GA	Gabon	MR	Mauritania	VN	Viet Nam

A METHOD FOR INCREASING TRANSDUCTION EFFICIENCY OF RECOMBINANT RETROVIRAL VECTORS

BACKGROUND OF THE INVENTION

To complete a retroviral life-cycle, the viral genomic
5 RNA carried by retroviral particles is reverse transcribed into
viral DNA, which is then integrated into the host chromosomal
DNA. The integrated proviral DNA then behaves as a residual
gene in the host cell's chromosome, participating in DNA
duplication during cell division and transcription of mRNA.
10 Based upon these characteristics, retroviral vectors have been
widely used to deliver foreign genes into target cells
(Kotani, H. et al. (1994) *Hum. Gene Ther.* 5:19-26; Miller, A.D.
and C. Buttimore (1986) *Mol. Cell. Biol.* 6:2895-2902; Miller,
A.D. and G.J. Rosman (1989) *Biotechniques* 7:980-990). However,
15 this delivery system still has a few problems.

One problem is the efficiency of reverse transcription
in certain target cells. Full-length retroviral DNA synthesis
is not detected in quiescent cells, partly due to the extremely
low deoxyribonucleoside triphosphate (dNTP) concentrations in
20 the quiescent cells, which are not sufficient to support
efficient reverse transcription (Gao, W.A. et al. (1993) *Proc.*
Natl. Acad. Sci. USA 90:8925-8928; Meyerhans, A. et al. (1994)
J. Virol. 68:535-540; Miller, D.G. et al. (1990) *Mol. Cell.*
Biol. 10:4239-4242; Zack, J.A. et al. (1990) *Cell* 61:214-222).
25 In addition, the compartmentalization of the dNTPs in certain
proliferating cells may also limit available dNTPs for viral
DNA synthesis (Leeds, J.M. et al. (1985) *Mol. Cell. Biol.*
5:3443-3450; Leeds, J.M. and C.K. Matthew (1985) *Mol. Cell.*

- 2 -

Biol. 7:532-534; Reddy, G.P.V. and R.S. Fager (1993) *Crit. Rev. Eukaryotic Gene Expr.* 3:255-277).

Endogenous reverse transcription in retroviruses is traditionally considered a somewhat artificial process which only mimics the reverse transcription occurring in the cytoplasm of target cells. Virion envelope permeabilization with reagents such as non-ionic detergents or melittin (a bee venom toxin) is routinely used for completion of the endogenous reverse transcription reaction (Boone, L.R. and A.M. Skalka 10 (1981) *J. Virol.* 37:117-126; Gilboa, E. et al. (1979) *Cell* 18:93-100; Rothenberg, E. and D. Baltimore (1976) *J. Virol.* 17:168-174). However, a certain level of reverse transcription can take place without detergent treatment of virions. This phenomenon was first demonstrated in the very early days of 15 retroviral study (Baltimore, D. (1970) *Nature* 226:1209-1211; Temin, H.M. and S. Mizutani (1970) *Nature* 226:1211-1213), and in the recent years this process was detected in studies of human immunodeficiency virus type 1 (HIV-1) virions (Borrito-Esoda, K. and L.R. Boone (1991) *J. Virol.* 65:1952-1959; 20 Debyser, Z. et al. (1992) *J. Biol. Chem.* 267:11769-11776; Yong, W.H. et al. (1990) *AIDS* 4:199-206; Zhang, H. et al. (1993) *AIDS Res. Hum. Retroviruses* 9:1287-1296). Some have credited this phenomenon to possible damage of the viral envelope during the process of virion purification or to freezing and thawing 25 resulting in artificial permeability of the virion envelope (Borrito-Esoda, K. and L.R. Boone (1991) *J. Virol.* 65:1952-1959; Debyser, Z. et al. (1992) *J. Biol. Chem.* 267:11769-11776).

However, examination of this phenomenon by different 30 methods, including initiation of reverse transcription with fresh virion-containing supernatants of infected cells prior to any isolation of viruses has demonstrated that this process is not dependent upon artificial permeabilization of the virion envelope (Zhang, H. et al. (1993) *AIDS Res. Hum. Retroviruses* 35 9:1287-1296). Further, HIV-1 virions treated with concentrated dNTPs initiate additional intravirion DNA synthesis and have been shown to then possess increased infectivity, as compared

- 3 -

to untreated virions (Zhang, H. et al. (1993) *AIDS Res. Hum. Retroviruses* 9:1287-1296).

It has now been found that the transduction efficiency of recombinant retroviral vectors is augmented by initiating
5 this endogenous reverse transcription prior to infection of target cells.

SUMMARY OF THE INVENTION

An object of the present invention is to provide a method for improving the transduction efficiency of retroviral
10 vectors into a host cell wherein the retroviral vectors are incubated with deoxyribonucleoside triphosphates prior to transduction into the host cell. Polyamines such as spermine, spermidine and putrescine and/or high concentrations of ribonucleoside triphosphates can also be added to the
15 incubation to improve transduction efficiency even further.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a schematic map of the murine retroviral vector (pLXSN-CAT), and shows the location of the primer pair for quantitative DNA polymerase chain reaction (PCR) analysis.
20 The 5' murine leukemia virus long terminal repeat (MLV-LTR) is used to express the chloramphenicol acetyl transferase (CAT) gene product, while the neomycin-resistance gene product (Neo^r) is expressed from an SV40 promoter.

Figure 2 is an autoradiograph showing the levels of
25 endogenous reverse transcription within recombinant murine retroviral virions that occurred when DNase-treated virions were mixed with deoxyribonucleoside triphosphates (dNTPs) at various concentrations.

Figure 3 is an autoradiograph showing quantitative
30 levels of proviral DNA in cells infected with the murine retroviral vector, the virions of which were treated with or without 5 mM deoxyribonucleoside triphosphates (dNTPs) prior to infection.

Figure 4 is a gel illustrating the CAT activity in
35 cells infected with the retroviral vector, the virions of which

- 4 -

were treated with or without 5 mM deoxyribonucleoside triphosphates (dNTPs) prior to infection.

DETAILED DESCRIPTION OF THE INVENTION

A method for increasing the transduction efficiency of retroviral vectors has been found. Prior to their use in infection, isolated, cell-free retrovirions are incubated in vitro with deoxyribonucleoside triphosphates (dNTPs) which allows them to complete reverse transcription of their RNA genomes. The resulting double-stranded heteroduplexed viruses have been demonstrated to have higher infectivity and be more efficient vectors in the transduction and expression of heterologous genes when transfected into appropriate cell lines. Polyamines such as spermine, spermidine and putrescine and/or high concentrations, i.e., 5 mM, of ribonucleoside triphosphates such as ATP, GTP, CTP and UTP can also be added to the incubations to further increase the transduction efficiency of recombinant retroviral vectors. The method of the present invention is markedly different from known methods for optimizing retroviral production and transduction (Kotani, H. et al. (1994) *Hum. Gene Ther.* 5:19-28). This method does not require any specialized procedures beyond standard virion purification methodologies. A quick and simple incubation of purified retrovirions with dNTPs is all that is required. In a preferred embodiment, the concentration of dNTPs used in eukaryotic cells is approximately 50 μ M. The addition of polyamines such as spermine, spermidine and putrescine and/or high concentrations, i.e., 5 mM, or ribonucleoside triphosphates such as ATP, GTP, CTP and UTP, in addition to the dNTPs in the incubation are believed to further increase the transduction efficiency of the retrovirions. The method of the present invention takes advantage of minor injuries to the virus which occur during routine purification and allow diffusion of the ionic nucleoside triphosphates through the normally impermeable envelopes of the virus. Furthermore, the method of the present invention does not require the additional steps of virion envelope permeabilization with non-ionic

- 5 -

detergents or melittin which may further disrupt viral membranes in a deleterious fashion. Nor is preparation of extremely high titer virus stocks required. The method of the present invention stands in marked contrast to and improves upon methods of increasing retroviral vector transduction efficiency using polycations, increasing viral titer, co-cultivation techniques or centrifugation of vector containing supernatants onto target cells. The method also does not require additional engineering of standard retroviral vectors, e.g., adding more tRNA primer binding sites (Lund, A.H. et al. (1993) *J. Virol.* 67:7125-7130).

The resulting reverse-transcribed viruses of the present invention also have kinetic advantages during infection of initially quiescent cells, in that the integratable double-stranded proviral DNA already exists prior to extracellular stimulation. Retroviral vectors stimulated artificially to complete endogenous reverse transcription have been shown to have higher infectivity. Thus, it is believed that the method of the present invention will also result in an increased efficiency of transduction and gene expression.

To demonstrate endogenous reverse transcription in recombinant MLV, a retroviral vector system was utilized as described by Duan, L. et al. in *Virol.* (1994) 199:474-478. PA317 cells, the packaging cell line which supplies the viral structural proteins (Miller, A.D. and C. Buttimore (1986) *Mol. Cell. Biol.* 6:2895-2902), were plated onto plates overnight. These cells were subsequently transfected with a plasmid containing the chloramphenicol acetyl transferase (CAT) gene. After 16 hours, the supernatant was removed, the cells were washed, and fresh medium was added. The supernatant was collected 48 hours after infection and cellular debris was removed by centrifugation. The virions were purified by centrifugation. The virion-containing suspension was then treated with RNase-free DNase. After incubation, the DNase was removed by isolating the virions. Endogenous reverse transcription was then initiated in a simple buffer system comprising Tris-HCl, NaCl, MgCl₂, and dNTPs at various

- 6 -

concentrations. The reaction was allowed to proceed for several hours at 37°C. The reaction was stopped and the viral DNA was extracted and amplified with the polymerase chain reaction (PCR).

5 The virion-associated DNA was first extracted with a lysing buffer prior to phenol-chloroform-isoamyl alcohol extraction and ethanol precipitation. After extraction, an aliquot of each sample was added an equivalent amount of a PCR mixture containing MgCl₂, dNTPs, positive- and negative-strand
10 primers, Taq DNA polymerase and Taq buffer. The samples were the subjected to 30 cycles of PCR consisting of 2 long denaturation cycles holding at 94°C for 3 minutes, decreasing to 53°C over a 3 minute period, and finally increasing to 72°C and holding there for 2 minutes. Following the first 2 cycles,
15 the remaining 28 cycles consisted of the same temperatures being held for 30 seconds each. The primer pair used to amplify the CAT gene was: CAT 01 (5'-GGAATGAAAGACGGTGAGCTG-3', nucleotides: 449-469 (SEQ ID NO: 1)); CAT 02 (5'-AGACGCCACATCTTGCGAATA-3', nucleotides: 581-601 (SEQ ID NO: 2)).
20 The PCR products were analyzed by Southern blot analysis, with a P 3 2 - l a b e l e d p r o b e , C A T 0 3 (5'-ACTGAAACGTTTTTCATCGCTCTGGAGTGAATAC-3', nucleotides 518-550 (SEQ ID NO: 3)) (Alton, N.K. and D. Vapnek (1979) *Nature* 282:864-869).

25 As demonstrated by Figure 2, there was some DNase-resistant viral DNA within the virions, even without addition of exogenous dNTPs (lane 1), which is consistent with previous findings (Lori, F. et al. (1992) *J. Virol.* 66:5067-5074; Trono, D. (1992) *J. Virol.* 66:4893-4900; Zhang, H. et al. (1993) *AIDS Res. Hum. Retroviruses* 9:1287-1296; Zhang, H. et al. (1994) *J. Virol.* 68:7591-7597; Zhu, J. and J.M. Cunningham (1993) *J. Virol.* 67:2385-2388). The *de novo* synthesized intravirion reverse transcripts were primarily expressed with the concentration of exogenous dNTPs reached a relatively high
30 level (5 mM) (Figure 2, lane 6). Serial dilutions of viral DNA, prepared prior to PCR, indicated that the increment of viral DNA driven by the dNTPs was approximately one hundred-

- 7 -

fold (Figure 2, lane 8). As expected (Borrito-Esoda, K. and L.R. Boone (1991) *J. Virol.* 65:1952-1959; Yong, W.H. et al. (1990) *AIDS* 4:199-206), more viral DNA was synthesized when the non-ionic detergent NP-10 was added at optimal concentrations
5 (Figure 2, lane 11).

To assess the functional impact of endogenous reverse transcription upon the infectivity of these recombinant MLV virions, the viruses were produced as described above. After elimination of cells and cellular debris by centrifugation, the
10 virion-enriched supernatants of virus-producing cells were incubated with $MgCl_2$, with or without 5 mM dNTPs, at 37°C for 2 hours. The virions were serially diluted and added into the wells of tissue culture plates containing NIH 3T3 cells per well with polybrene. After incubation for 16 hours at 37°C,
15 the cells were vigorously washed three times with PBS, and fresh DMEM containing FCS was added to the cultures. Thereafter, the viral infectivity was measured via three complementary assays: 1) quantitative PCR for proviral DNA copy number within infected cells, 2) assay of colony forming
20 units (cfu), and 3) CAT assays for expression of the transduced gene.

Quantitative PCR analysis of proviral DNA in the target cells, after 14 days, indicated that more proviral DNA was synthesized in the cells when the input viruses were
25 treated with dNTPs, as compared to untreated virions. By either copy number analysis or end-point estimation, this increase was approximately 100-fold (Figure 3). Further, the expression of the two reporter genes in the retroviral vector was approximately 10-fold higher when the input virions were
30 treated with dNTPs, as measured by either CAT assays or cfu analyses (Figure 4 and Table I). The percent conversions of chloramphenicol in the CAT assays were approximately 20% and 2% for undiluted virions, treated and untreated with dNTPs, respectively (Figure 4, lanes 1 and 2).

TABLE 1

Comparison of Transduction Efficiencies of Murine Leukemia Virus (MLV)-Vectors After Treatment With and Without Deoxyribonucleoside Triphosphates (dNTPs)

	+dNTPs (5 mM)	-dNTPs
5 First Experiment	6.4×10^6 cfu	6.8×10^5 cfu
Second Experiment	4.5×10^6 cfu	5.0×10^5 cfu

As illustrated in Figure 1, the CAT gene product was expressed by the 5' MLV-LTR, while the neomycin-resistance
10 (neo^r) gene was driven by an SV40 promoter. Thus, their expression should be independent of each other. The increased expression of both genes, at the same level, suggests that the copy number of their proviral DNA was increased. It was found that the increment for the expression of two reporter genes is
15 less than that for the proviral DNA synthesis in the target cells. It is believed that this difference is due to viral DNA synthesized within the virions which is not suitable to integrate into the host cell's chromosomal DNA.

The retroviral envelope is derived from the cellular
20 membrane of virus-producing cells and, therefore, it would be presumed that the viral envelope is not permeable to dNTPs. Nevertheless, the data presented here indicates that endogenous reverse transcription for the recombinant murine leukemia virus occurs only in the presence of dNTP. A similar phenomenon has
25 been demonstrated in another type C retrovirus, human T-cell leukemia/lymphoma virus type I (HTLV-1).

The products of the method of retroviral endogenous reverse transcription of the present invention may directly integrate into chromosomal DNA and/or take a shorter time to
30 complete reverse transcription as compared to virions which solely harbor genomic RNA (Zhang, H. et al. (1993) *AIDS Res. Hum. Retroviruses* 9:1287-1296; Zhang, H. et al. (1994) *J. Virol.* 68:7591-7597). Further, a virion-derived structure, the

- 9 -

nucleoprotein complex, may be necessary for viral DNA synthesis, especially for efficient template switching during reverse transcription (Bowerman, B. et al. (1989) *Genes & Develop.* 3:469-478; Bukrinsky, M.I. et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:6125-6129; Coffin, J.M. (1990) *Virology*, B.N. Fields (ed.), Raven Press Ltd., New York, pp. 1437-1499). If this replicative machinery remains in a target cell for a significant time, it may begin to degrade prior to completion of reverse transcription. Therefore, those viruses that enter non-S phase cells harboring relatively low concentrations of dNTPs in the cytoplasm can gain significant benefit from the reverse transcription before entering these target cells.

These present invention provides a quick, simple and efficient method to increase the transduction efficiency of many retroviral vectors. One of skill in the art, upon reading this disclosure, can routinely implement this efficiency augmentation process to any retroviral vector and ascertain its success. Examples of retrovirus-based vectors include, but are not limited to, murine leukemia virus-derived vectors, lentivirus-based vectors such as HIV-1 derived vector, and avian leukemia virus-based vectors such as spleen necrosis virus. Retroviral vectors are routinely used by those of skill in the art for the transduction of a gene into a host cell. The transduced cells can then be used for production and isolation of many different proteins including, but certainly not limited to, cytokines, interleukins and enzymes. The method of the present invention improves this transduction process, thus facilitating the production of such proteins. In addition, retroviral vectors have been proposed for use in gene therapy. For example, see WO 9418995 disclosing retroviral transformed tumor cells for producing immuno-molecules which are used in the treatment and prevention of the formation of malignant tumors and WO 9112329 disclosing a method for the *in vivo* inclusion of a foreign gene into an adult eukaryotic tissue by infecting a mitotically-active cell in the tissue with a retroviral vector. It is believed that the method of the present invention can also be used in specific gene

- 10 -

therapeutic techniques such as those exemplified. Transduction efficiency of a viral vector can be largely increased when the target cells are at a stationary stage. Accordingly, it is believed that the method of the present invention will be especially useful to conduct foreign genes into hematopoietic stem cells, peripheral blood mononuclear cells, monocytes or macrophages and endothelial cell.

The following nonlimiting examples are provided to further illustrate the present invention.

10 EXAMPLES

Example 1: Preparation of Recombinant MLV vector system

PA317 cells (1×10^6) were plated onto 100 mm plates overnight. These cells were subsequently transfected with 50 μ g of a plasmid containing the chloramphenicol acetyl transferase (CAT) gene driven from the MLV long terminal repeat (LTR) in an MLV backbone, pLXSN-CAT (Duan, L. et al. (1994) *Virol.* 199:474-478; Miller, A.D. and G.J. Rosman (1989) *Biotechniques* 7:980-990), (Figure 1) using the calcium-phosphate precipitation system as described by Kingston, R.E. (1992) in *Current Protocols in Molecular Biology*, 9.1.1 - 9.1.3. After 16 hours, the supernatant was removed, the cells were washed with phosphate-buffered saline (PBS), and fresh Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal calf serum (FCS) was added. The supernatant was collected 48 hours after infection and cellular debris was removed by centrifugation at 300 x g for 10 minutes, followed by another at 5,000 x g for 10 minutes. The virions were purified by centrifugation onto a 20% sucrose cushion at 60,000 x g for 40 minutes in a Beckman Ti 50 rotor (Beckman Instruments, Inc., Fullerton, CA) at 4°C. The virion-containing suspension (1 ml) was then treated with 40 Units of RQ1 RNase-free DNase (Promega, Madison, WI). After 1 hour incubation at 37°C, the DNase was removed by isolating the virions on the sucrose cushion above. Endogenous reverse transcription was then initiated in a simple buffer system: 10 mM Tris-HCl (pH 7.6), 100 mM NaCl, 2.4 mM MgCl₂, and dNTPs

- 11 -

(Pharmacia, Piscataway, NJ) at various concentrations. The reaction was allowed to proceed for 2 hours at 37°C. After boiling for 10 minutes to stop the reaction, the viral DNA was extracted and amplified with the polymerase chain reaction (PCR).

Example 2: Measuring Endogenous Reverse Transcription

The virion-associated DNA was extracted with a lysing buffer (10 mM Tris-HCl [pH 7.6], 100 mM NaCl, 1% sodium dodecyl sulfate [SDS], proteinase K [100 mg/ml], prior to phenol-chloroform-isoamyl alcohol (24:24:1) extraction and ethanol precipitation. After extraction, 25 ml of samples were added to 25 ml of a PCR mixture (5 mM MgCl₂, 220 mM dNTPs, 50 pmole of positive- and negative-strand primers, 2 Units of Taq DNA polymerase [Perkin-Elmer Cetus, Foster City, CA], 5 ml of Taq buffer). The samples were subjected to 30 cycles of PCR consisting of 2 long denaturation cycles holding at 94°C for 3 minutes, decreasing to 53°C over a 3 minute period, and finally increasing to 72°C and holding there for 2 minutes. Following the first 2 cycles, the remaining 28 cycles consisted of the same temperatures being held for 30 seconds each. The reactions were performed on an automated DNA thermocycle (Perkin-Elmer Cetus, Foster City, CA). The primer pair used to amplify the CAT gene was: CAT 01 (5'-GGAATGAAAGACGGTGAGCTG-3', nucleotides: 449-469 (SEQ ID NO: 1)); CAT 02 (5'-AGACGCCACATCTTGCGAATA-3', nucleotides: 581-601 (SEQ ID NO: 2)). The PCR products were separated on a 1.5% agarose gel and transferred to a nylon filter. The products were analyzed by Southern blot analysis, with a P32-labeled probe, CAT 03 (5'-ACTGAAACGTTTTTCATCGCTCTGGAGTGAATAC-3', nucleotides 518-550 (SEQ ID NO: 3)) (Alton, N.K. and D. Vapnek (1979) Nature 282:864-869) (Figure 2, lanes 1-11).

As a positive control and as a standard curve, a serial dilution of known copy numbers of the chloramphenicol acetyl transferase (CAT)-expressing plasmid (pLXSN-CAT) was analyzed under the same condition (Figure 2, lanes 12-17). The experiments were done in independent duplicates.

- 12 -

Example 3: Measuring Infectivity

To assess the functional impact of endogenous reverse transcription upon the infectivity of these recombinant MLV virions, the viruses were produced as described in Example 2. After elimination of cells and cellular debris by sequential 10 minutes centrifugations at 500 x g and 5,000 x g, the virion-enriched supernatants of virus-producing cells were treated with 2.4 mM MgCl₂, with or without 5 mM dNTPs, at 37°C for 2 hours. The virions were serially diluted, by 10-fold increments, and added into the wells of 24-well tissue culture plates containing 4 X 10⁴ NIH 3T3 cells per well, with 5 mg/ml polybrene. After incubation for 16 hours at 37°C, the cells were vigorously washed three times with PBS, and fresh DMEM plus 10% FCS was added to the cultures. Thereafter, the viral infectivity was measured via three complementary assays: 1) quantitative PCR for proviral DNA copy number within infected cells, 2) assay of colony forming units (cfu), and 3) CAT assays for expression of the transduced gene.

For quantitative PCR assays to detect proviral DNA in infected cells, cells were replated at 20% confluency every 3 to 4 days post-infection. Fourteen days later, the cells were collected, the DNA was extracted and proviral DNA was amplified using PCR as described in Example 2.

For the colony forming unit assay, G418 (400 mg/ml, active, GIBCO-BRL, Gaithersburg, MD) was added to the cultures, twenty-four hours post-infection. The G418-resistant cfu were then counted by light microscopy after 14 days.

For CAT analysis, the cells were washed with PBS three days post-infection, and then collected with a rubber cell-scraper in 300 ml of 250 mM Tris-HCl (pH 7.8). CAT assays were performed in accordance with procedures described by Gorman, C. et al. (1982) *Mol. Cell. Biol.* 2:1044-1051.

- 13 -

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Hui Zhang, Roger J. Pomerantz
- (ii) TITLE OF INVENTION: A Method for Increasing
Transduction Efficiency of Recombinant
Retroviral Vectors
- (iii) NUMBER OF SEQUENCES: 3
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Jane Massey Licata, Esq.
 - (B) STREET: 210 Lake Drive East, Suite 201
 - (C) CITY: Cherry Hill
 - (D) STATE: NJ
 - (E) COUNTRY: USA
 - (F) ZIP: 08002
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: DISKETTE, 3.5 INCH,
1.44 Mb STORAGE
 - (B) COMPUTER: IBM 486
 - (C) OPERATING SYSTEM: WINDOWS FOR WORKGROUPS
 - (D) SOFTWARE: WORDPERFECT 5.1
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: not yet assigned
 - (B) FILING DATE: Herewith
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: 08/385,446
 - (B) FILING DATE: February 8, 1995
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Jane Massey Licata

- 14 -

(B) REGISTRATION NUMBER: 32,257

(C) REFERENCE/DOCKET NUMBER: JEFF-0153

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (609) 779-2400

(B) TELEFAX: (609) 779-8488

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: No

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GGAATGAAAG ACGGTGAGCT G 21

2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: No

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

AGACGCCACA TCTTGCGAAT A 21

2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

- 15 -

(D) TOPOLOGY: Linear.

(iv) ANTI-SENSE: No

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

ACTGAAACGT TTTCATCGCT CTGGAGTGAA TAC

33

- 16 -

What is claimed is:

1. A method for improving the transduction efficiency of retroviral vectors into a host cell comprising incubating a retroviral vector with deoxyribonucleoside triphosphates 5 prior to transduction into the host cell.

1/4

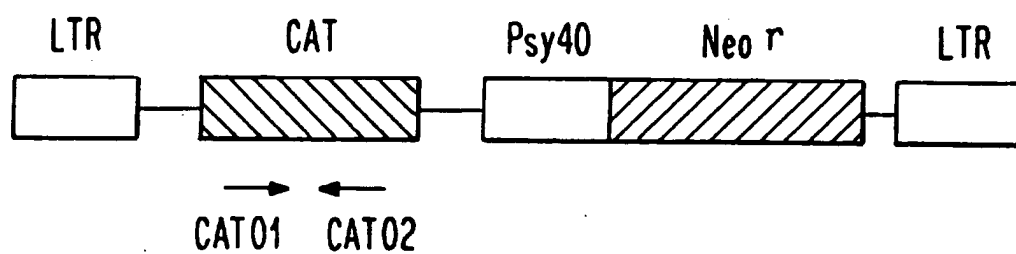
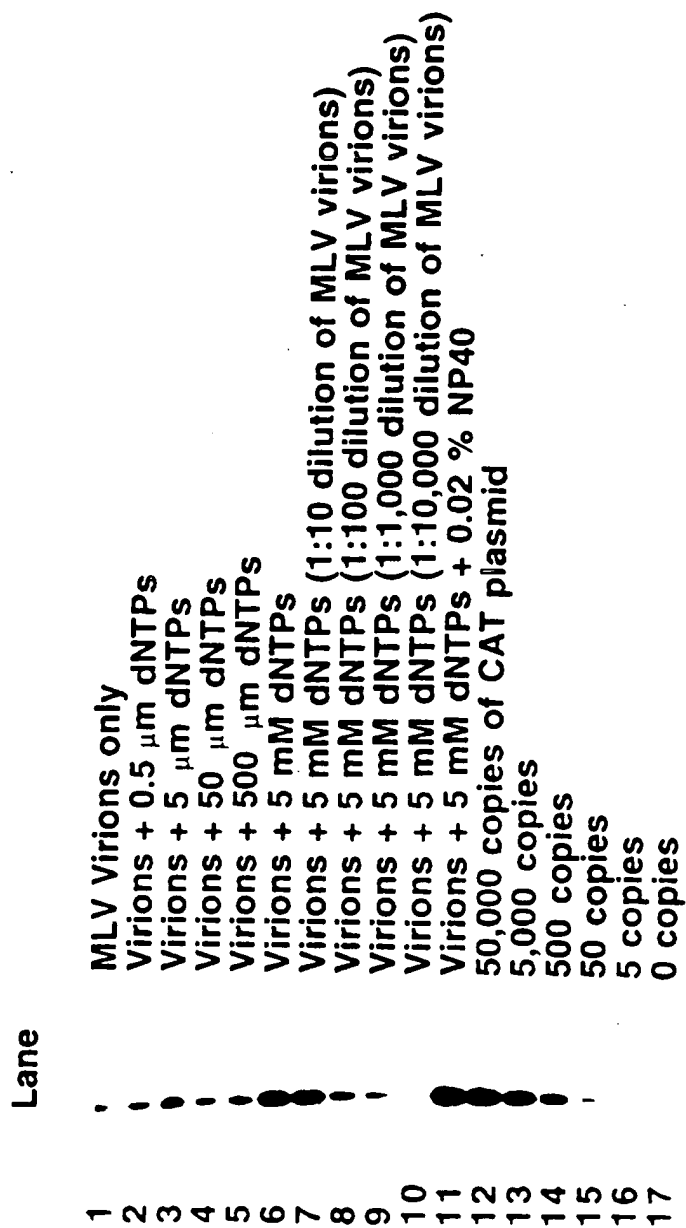


Fig. 1

2/4



CAT 01 / CAT 02 Primers

FIG. 2

3/4

Virus Input	
1:10	with dNTPs
1:100	
1:1,000	
1:10,000	
1:100,000	
1:1,000,000	
1:10	without dNTPs
1:100	
1:1,000	
1:10,000	
1:100,000	
1:1,000,000	
5,000 copies	
500 copies	
50 copies	
5 copies	
0 copies	

CAT01/CAT02

FIG. 3

4/4

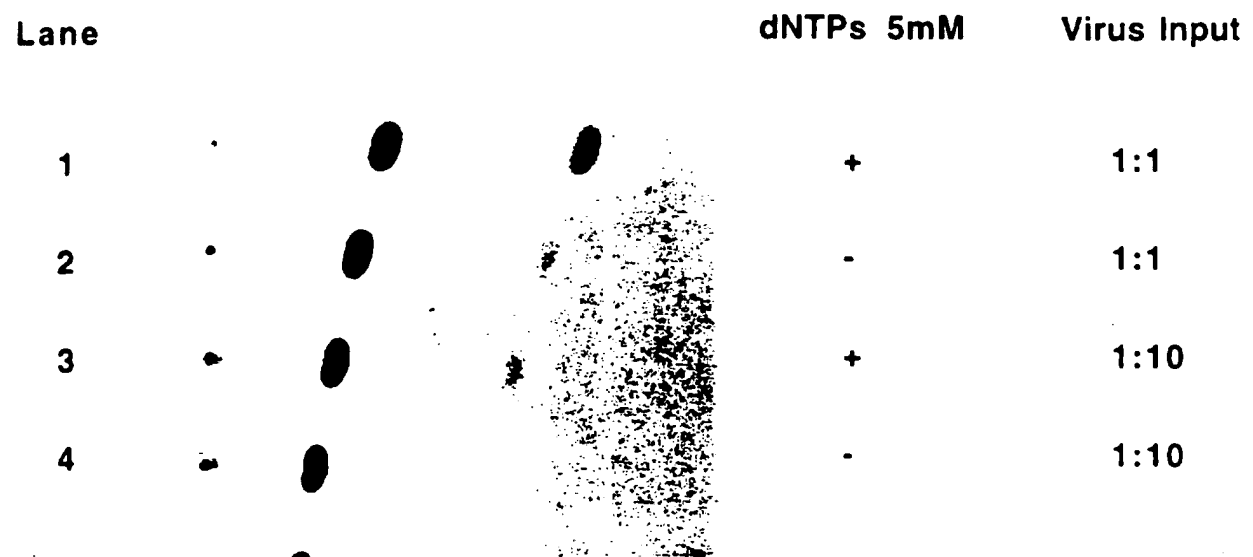


FIG. 4

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/01958

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 15/64, 15/86, 15/63

US CL : 435/320.1, 235.1; 514/43

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/320.1, 235.1; 514/43

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG DATABASE: MEDLINE, BIOSIS PREVIEWS, AIDSLINE, WORLD PATENT INDEX, CA SEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PAGE et al. Construction and Use of a Human Immunodeficiency Virus Vector for Analysis of Virus Infectivity. Journal of Virology. November 1990, Vol. 64, No. 11, pages 5270-5276, see entire article.	1
Y	ZHANG et al. Reverse Transcription Takes Place within Extracellular HIV-1 Virions: Potential Biological Significance. AIDS Research and Human Retroviruses. 1993, Vol. 9, No. 12, pages 1287-1296, see entire article.	1

☐

Further documents are listed in the continuation of Box C.

☐

See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z*	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

20 MAY 1996

Date of mailing of the international search report

30 MAY 1996

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Authorized officer

JOHNNY F. RAILEY II, PH.D.

Facsimile No. (703) 305-3230

Telephone No. (703) 308-0196